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Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes

(B-cell proliferation/immunoglobulin production)

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ABSTRACT **Interleukin 10 (IL-10), originally identified as a T_H2 helper T-cell product able to inhibit cytokine production by T_H1 cells, is highly homologous to BCRF1 (viral IL-10), an open reading frame in the Epstein–Barr virus genome. Here, we show that human and viral IL-10 stimulate DNA replication of B lymphocytes activated either via their antigen receptor or via their CD40 antigen. IL-4 and IL-10 display additive effects and induce a strong increase in the number of viable cells. Moreover, IL-10 induces activated B cells to secrete large amounts of IgG, IgA, and IgM, and the combination of IL-10 and IL-4 results in the secretion of the four immunoglobulin isotypes. Thus, IL-10 may play an important role in the amplification of humoral responses.**

Both the nature and route of administration of antigen strongly influence the class of the immune response to that antigen. Recently, a subdivision between T_H1 and T_H2 cells was proposed according to the panel of cytokines secreted by long-term mouse (1, 2) and human (3, 4) helper T-cell clones. T_H1 cells, producing interleukin 2 (IL-2) and interferon-γ, are associated with some types of delayed-type hypersensitivity (5), whereas T_H2 clones, producing IL-4 and IL-5, are associated with humoral responses (6). Furthermore, T_H2 cells have been found to specifically produce a cytokine inhibiting the synthesis of several cytokines by T_H1 clones. This cytokine, first called cytokine synthesis inhibitory factor (CSIF), has since been renamed IL-10 (7–9).

Mouse and human IL-10 display a high sequence similarity with an open reading frame in the Epstein–Barr virus (EBV), BCRF1 (8), coding for a putative protein expressed in the late viral lytic cycle (ref. 10; G. S. Hudson, D.-H.H., and K.W.M., unpublished results). Even if the product of the BCRF1 gene, viral IL-10 (vIL-10), does not display all the properties described for IL-10, it has retained from cellular IL-10 the capacity to block cytokine synthesis, a function that could give a selective advantage to EBV (7, 9, 11, 12). A common feature shared by T_H2 cells and EBV is their capacity to stimulate B-cell activation, proliferation, and antibody production (1, 2, 6, 13). Therefore, we speculated that aspects of this phenomenon might be mediated by both types of IL-10. Accordingly, we studied the effects of recombinant human IL-10 (hIL-10) and vIL-10 on the proliferation and differentiation of human B cells activated via their antigen receptors (surface immunoglobulin) or via their CD40 antigen (14, 15).

MATERIALS AND METHODS

Reagents. The anti-CD40 monoclonal antibody mAb89 was produced in the laboratory (16). The CDw32/Fc_γ receptor II-transfected thymidine kinase-negative L-cell line (CDw32 L cells) was described earlier (17). Formalin-fixed *Staphylococcus aureus* strain Cowan (SAC) (Pansorbin) was from Calbiochem, and anti-IgM antibodies coupled to beads (anti-μ) were purchased from Bio-Rad. Cell phenotype was determined using fluorescein-conjugated monoclonal antibodies from Becton Dickinson. Cultures were incubated in modified Iscove's medium as detailed previously (18).

B-Cell Preparations and Cell Cultures. B cells were isolated from tonsils as previously described (19). Briefly, after a rosetting step with sheep red blood cells, non-rosetting cells were further incubated with anti-CD2, anti-CD3, and anti-CD14 monoclonal antibodies prior to negative selection performed with magnetic beads coated with anti-mouse IgG (Dynabeads, Dynal, Oslo). In the isolated cell population, >98% expressed CD19 or CD20 (B cells) and <1% expressed CD2 (T cells) or CD14 (monocytes).

Assays for preactivated B cells. B lymphocytes, adjusted to 1×10^6 cells per ml, were stimulated for 48 hr by insolubilized anti-μ (5 μg/ml) or SAC (0.01% final dilution) as described (19). B blasts were dispensed at 5×10^4 cells per well in 100 μl. For proliferation assays, cells were incubated with [^{3}H]thymidine [1 μCi (37 kBq) per well] for 16 hr, usually at days 3 and 6. [^{3}H]Thymidine incorporation was measured by standard liquid scintillation counting techniques. For determination of immunoglobulin production, culture supernatants were harvested at day 5 and IgG, IgM, IgA, and IgE were measured in standard ELISAs (18).

CD40 system. For proliferation assays, 2.5×10^4 purified B cells were cultured in the presence of 2.5×10^3 irradiated [7000 rads (70 Gy)] CDw32 L cells and anti-CD40 mAb89 (0.5 μg/ml) in a final volume of 100 μl (14, 15). Cells were generally harvested at days 3 and 7. For immunoglobulin production, 0.5 ml of B cells at 2×10^5 per ml were cultured in 48-well plates with 10^4 irradiated CDw32 L cells and mAb89 (0.5 μg/ml). Supernatants were harvested after 10 days and immunoglobulin levels were determined by ELISA.

ELISPOT Assay for Immunoglobulin Production by Single Cells. The ELISPOT assay is adapted from the technique described by Czerkinsky *et al.* (20). To detect immunoglobulin-producing cells, plates were coated for 2 hr at 37°C with rabbit anti-human immunoglobulin heavy-chain antiserum in 0.05 M carbonate/bicarbonate buffer (pH 9.6). Anti-IgM, -IgG, and -IgA antisera (Behring Institut, Marburg, F.R.G.) were used at 1:1000, 1:2000, and 1:10,000 dilution, respectively. After washing, serial dilutions (from 30,000 to 30 cells per well) of anti-CD40-activated B-cell cultures and control cell lines, diluted in RPMI-1640/10% fetal bovine serum, were dispensed in triplicate in a final volume of 100 μl and incubated for 2 hr at 37°C in a 5% CO_2 atmosphere. Biotinylated

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Abbreviations: EBV, Epstein–Barr virus; IL, interleukin; hIL, human IL; vIL, viral IL; SAC, *Staphylococcus aureus* strain Cowan.

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Table 1. IL-10 enhances DNA synthesis of B cells activated via surface immunoglobulin

IL-2/4	IL-10	[^{3}H]Thymidine incorporation, cpm × 10^{-3} NA	SAC	Anti-μ
–	–	0.7 ± 0.1	3 ± 0.3	2 ± 0.3
	hIL-10	0.6 ± 0.1	12 ± 1.0	10 ± 0.8
	vIL-10	0.6 ± 0.1	13 ± 0.6	12 ± 1.0
IL-2	–	0.3 ± 0.1	48 ± 1.2	28 ± 2.0
	hIL-10	0.4 ± 0.1	45 ± 2.7	29 ± 2.0
	vIL-10	0.7 ± 0.2	48 ± 3.1	27 ± 2.3
IL-4	–	0.6 ± 0.1	20 ± 1.0	31 ± 1.9
	hIL-10	0.8 ± 0.1	21 ± 2.2	35 ± 3.7
	vIL-10	0.6 ± 0.1	20 ± 2.2	34 ± 3.0

Purified B cells were preactivated for 48 hr with either anti-μ beads or SAC particles. NA, not activated. hIL-10 and vIL-10 were used at 10 ng/ml as COS-7 transfectant supernatants. Control supernatant from mock-transfected COS-7 cells was inactive. IL-2 and IL-4 were used at 10 and 50 units/ml, respectively. Cells were harvested at day 3. Results are means ± SD of triplicates.

Table 2. IL-10 strongly enhances DNA synthesis of B cells activated through their CD40 antigen

IL-2/4	IL-10	[^{3}H]Thymidine incorporation, cpm × 10^{-3} mAb89	CDw32 L cell	mAb89 + CDw32 L cell
–	–	0.2 ± 0.1	1.2 ± 0.2	14 ± 1.4
	hIL-10	0.2 ± 0.1	1.0 ± 0.1	98 ± 5.6
	vIL-10	0.3 ± 0.1	1.2 ± 0.3	107 ± 8.8
IL-2	–	0.3 ± 0.1	1.4 ± 0.2	15 ± 0.9
	hIL-10	0.3 ± 0.1	1.2 ± 0.4	95 ± 7.8
	vIL-10	0.2 ± 0.1	1.4 ± 0.4	104 ± 9.2
IL-4	–	0.6 ± 0.1	1.5 ± 0.3	104 ± 3.7
	hIL-10	0.4 ± 0.1	1.9 ± 0.4	205 ± 11.0
	vIL-10	0.9 ± 0.1	2.2 ± 0.1	221 ± 9.7

Purified B cells were cultured on CDw32 L cells, in the presence of mAb89 (0.5 μg/ml), or both. hIL-10 and vIL-10 were used at 10 ng/ml as COS-7 transfectant supernatants. Control supernatant was inactive. IL-2 and IL-4 were used at 10 and 50 units/ml, respectively. Cells were harvested at day 7. Results are means ± SD of triplicates.

species-specific polyclonal sheep antibody against human immunoglobulin (Amersham), diluted 1:1000, was then added. After 2 hr of incubation at room temperature, plates were washed and incubated for 2 hr with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch) diluted 1:600. Finally, 100 μl of a prewarmed solution of 5-bromo-4-chloro-3-indolyl phosphate (Sigma) dissolved at 1 mg/ml in 0.6% agarose (BRL) was added. Spots were enumerated 12 hr after addition of substrate. The optimum number of spots that could be counted per microwell was between 10 and 70. Frequencies were calculated as the ratio of the number of spots to the number of cells plated per microwell. Results are expressed as means ± SD of triplicate determinations.

Cytokines. Purified recombinant hIL-2 (Amgen; 3 × 10^6 units/ml) and hIL-4 (Schering–Plough; 10^7 units/mg) were used at 10 and 50 units/ml, respectively. Recombinant hIL-10 and vIL-10 or their respective mock controls were culture supernatants from COS-7 transfectants. Purified recombinant h/vIL-10 from *Escherichia coli* material was obtained as described by de Waal Malefyt *et al.* (12). Based on an IL-10-specific ELISA, COS-7 transfectant supernatants contained IL-10 at 0.6–1.2 μg/ml and, for comparable concentrations, gave biological activities similar to those generated with the purified *E. coli*-derived material.

RESULTS

hIL-10 and vIL-10 Are Potent Growth Factors for Activated B Lymphocytes. Recombinant hIL-10 and vIL-10, in the form of COS-7 transfectant supernatants or *E. coli*-derived purified proteins, were added to highly purified human B lymphocytes activated via surface immunoglobulin (SAC or anti-μ) or via CD40 antigen (anti-CD40 mAb89 and CDw32-transfected mouse L cells) (14, 15). As shown in Table 1, hIL-10 as well as vIL-10 enhanced [^{3}H]thymidine incorporation of human B lymphocytes activated through ligation of surface immunoglobulin by SAC or anti-μ, whereas it was ineffective on B cells cultured without polyclonal activator. Dose–response analysis with pure products (data not shown) indicated that the two cytokines displayed stimulating effects with similar amplitude. Yet even at optimal concentrations, the h/vIL-10-induced levels of [^{3}H]thymidine incorporation were always lower than those mediated by IL-2 or IL-4.

When B cells were activated in the CD40 system, h/vIL-10 showed a strong costimulatory effect on DNA replication (Table 2). In these culture conditions, the B-cell growth-promoting effects of IL-10 and IL-4 were comparable, whereas IL-2 was virtually inactive. The proliferative effects of purified *E. coli*-derived hIL-10 and vIL-10 were dose-dependent and comparable (Fig. 1*A*). IL-10 (both human and viral) acted in combination with IL-4 to induce strong B-cell growth. In both cases, a plateau was reached at 10 ng/ml. As demonstrated by kinetic studies (Fig. 1*B*), the B-cell growth effects of IL-4 and h/vIL-10, which started at day 3, were quite comparable until day 7, after which DNA replication slowed down with IL-10 but not with IL-4. Moreover, from

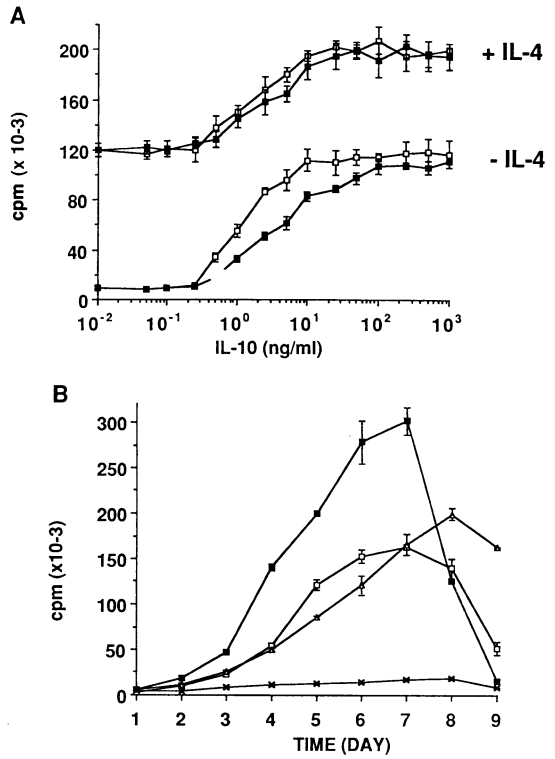

FIG. 1. IL-10 potentiates DNA replication of anti-CD40-activated B lymphocytes. (*A*) Anti-CD40-activated B cells were cultured with purified *E. coli*-derived recombinant hIL-10 (■) and vIL-10 (□) in the absence or presence of IL-4 (50 units/ml). [^{3}H]Thymidine incorporation was assayed at day 7. (*B*) Anti-CD40-crosslinked activated B cells were cultured with IL-10 (10 ng/ml; COS-7 transfectant supernatant) and/or IL-4 (50 units/ml). Culture supernatant from mock-transfected COS-7 cells was used as control and was inactive. Kinetic curves show [^{3}H]thymidine incorporation without addition of any cytokine (×), with IL-4 (□), with hIL-10 (△), and with IL-4 plus hIL-10 (■). Similar kinetics were obtained with vIL-10. Results are means ± SD of triplicates.

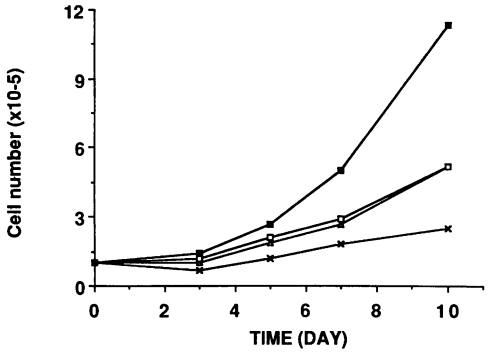

FIG. 2. IL-10 is a growth factor for anti-CD40-activated B lymphocytes. Cells were cultured in the CD40 system without any cytokine (×), with IL-4 (50 units/ml; □), with vIL-10 (10 ng/ml; COS-7 transfectant supernatant; △), and with IL-4 plus vIL-10 (■). Similar results were obtained with hIL-10. Samples were harvested at days 3, 5, 7, and 10 and viable cells were enumerated after trypan blue staining.

day 3 to day 7, IL-4 and h/vIL-10 clearly displayed additive effects when used simultaneously. Cell counts, performed at different time points in an overall culture period of 10 days (Fig. 2), unequivocally confirmed that the [^{3}H]thymidine incorporation observed in response to h/vIL-10 reflected B-cell proliferation. h/vIL-10 appeared to be as efficient as IL-4 in increasing the number of B lymphocytes in the CD40 system and resulted in a 4- to 5-fold multiplication of the input B cells within 10 days. Flow cytometry demonstrated that cells proliferating in response to h/vIL-10 expressed CD19 and CD20 (data not shown). Combination of IL-4 and h/vIL-10 resulted in an ≈10-fold expansion of the number of viable B cells within 10 days. These results indicate that hIL-10 and its viral homolog are potent growth factors for purified, activated human B cells.

hIL-10 and vIL-10 Stimulate Immunoglobulin Production of Activated B Lymphocytes. The activity of h/vIL-10 in B-cell differentiation was examined next. As shown in Table 3, IL-10 induced SAC-preactivated B lymphocytes to produce high levels of IgM, IgG, and IgA. hIL-10 and vIL-10 gave similar results. Moreover, IL-10 appeared to be more potent than either IL-2 or IL-4, cytokines considered, until now, as the most efficient in this culture system (21).

When B lymphocytes were activated in the CD40 system, addition of IL-10 resulted in the production of considerable amounts of IgM, IgG, and IgA (Table 4). As observed with SAC-activated cells, IL-10 was much more efficient than either IL-2 or IL-4. The IL-10-induced immunoglobulin production was dose-related, with maximal immunoglobulin production and cell proliferation obtained for the same h/vIL-10 concentrations (data not shown). Unlike IL-4, neither vIL-10 nor hIL-10 induced anti-CD40-activated B

Table 3. IL-10 stimulates immunoglobulin production of SAC-preactivated B cells

Cytokine	Immunoglobulin production, μg/ml		
	IgM	IgG	IgA
–	0.09 ± 0.01	0.8 ± 0.06	0.1 ± 0.01
IL-2	0.6 ± 0.05	4.5 ± 0.4	0.2 ± 0.05
IL-4	0.8 ± 0.09	2.1 ± 0.1	0.1 ± 0.01
hIL-10	2.9 ± 0.1	6.9 ± 0.3	1.1 ± 0.07
vIL-10	3.5 ± 0.2	6.6 ± 0.8	0.6 ± 0.01

Purified B cells were preactivated for 48 hr with SAC particles. hIL-10 and vIL-10 were used at 10 ng/ml as COS-7 transfectant supernatants. Control supernatant was inactive. IL-2 and IL-4 were used at 10 and 50 units/ml, respectively. Supernatants were harvested at day 5 and immunoglobulin levels were determined by ELISA. Results are means ± SD of triplicate determinations.

Table 4. IL-10 stimulates immunoglobulin production of B cells activated through their CD40 antigen

Cytokine(s)	Immunoglobulin production			
	IgM, μg/ml	IgG, μg/ml	IgA, μg/ml	IgE, ng/ml
No IL-2/4				
No IL-10	0.1 ± 0.01	0.4 ± 0.03	0.04 ± 0.01	<0.3
hIL-10	11.1 ± 0.5	10.5 ± 0.4	4.0 ± 0.2	<0.3
vIL-10	16.9 ± 0.9	13.8 ± 0.1	5.2 ± 0.4	<0.3
IL-2				
No IL-10	0.08 ± 0.01	0.4 ± 0.03	0.03 ± 0.01	<0.3
hIL-10	12.2 ± 1.1	11.8 ± 0.9	3.5 ± 0.3	<0.3
vIL-10	14.0 ± 1.3	13.2 ± 0.5	4.4 ± 0.6	<0.3
IL-4				
No IL-10	0.5 ± 0.06	1.0 ± 0.08	0.1 ± 0.01	127 ± 5.0
hIL-10	9.8 ± 1.0	7.2 ± 0.4	1.7 ± 0.2	145 ± 3.0
vIL-10	10.3 ± 1.2	6.9 ± 0.8	2.8 ± 0.4	194 ± 11.0

Purified B cells were cultured on CDw32 L cells with mAb89 (0.5 μg/ml). hIL-10 and vIL-10 were used at 10 ng/ml as COS-7 transfectant supernatants. Control supernatant was inactive. IL-2 and IL-4 were used at 10 and 50 units/ml, respectively. Supernatants were harvested at day 10 and immunoglobulin levels were determined by ELISA. Results are means ± SD of triplicate determinations.

lymphocytes to secrete IgE. The four isotypes were detected when IL-4 and IL-10 were added simultaneously. However, whereas the combination of IL-4 and IL-10 had additive effects on B-cell proliferation, the levels of secreted IgM, IgG, and IgA were lower than those observed with IL-10 alone (Table 4). Addition of IL-4 at only 1 unit/ml resulted in a 50% decrease of the IL-10-induced IgM, IgG, and IgA production (Fig. 3: only IgM results, representative of the three isotypes, are shown); maximal inhibition (75%) was obtained with IL-4 at 10 units/ml. Addition of IL-10 only weakly affected IL-4-induced IgE production. A large proportion of anti-CD40-activated B cells secreted immunoglobulin in response to IL-10 (Table 5). Thus, 40% of cells secreted IgM, 43% secreted IgG, and 6% secreted IgA. In contrast only 10% of cells cultured in the presence of IL-4 secreted IgG, and the size of the IL-4-induced spots was considerably smaller than that observed with IL-10 (data not shown). As observed for immunoglobulin levels in supernatants, IL-4 decreased the frequency of IL-10-induced immunoglobulin-secreting cells.

DISCUSSION

We have demonstrated that hIL-10 and the highly related viral protein vIL-10 can induce both proliferation and differ-

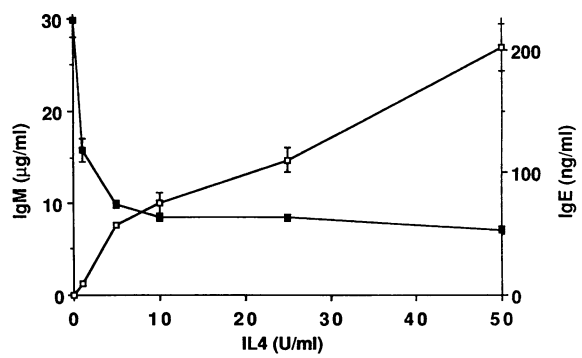

FIG. 3. IL-4 inhibition of IL-10-induced IgM production is dose-dependent. Anti-CD40-activated B lymphocytes (10^5) were cultured for 10 days in the presence of vIL-10 (10 ng/ml; COS-7 transfectant supernatant). Various doses of IL-4 [units (U)/ml] were simultaneously added. Supernatants were harvested and their IgM, IgG, IgA, and IgE levels determined. Only IgM (■) and IgE (□) data are shown here. Comparable results were obtained with hIL-10. Results are means ± SD of culture triplicates.

Table 5. IL-10 induces a high proportion of anti-CD40-activated B cells to secrete immunoglobulin

Cytokine(s)	Immunoglobulin-secreting cells, % total cells		
	IgM	IgG	IgA
None	2 ± 1	2 ± 0	<0.5
IL-4	1.7 ± 0.5	10 ± 1.6	<0.5
hIL-10	40 ± 4	43 ± 3.3	6 ± 1.4
IL-4 + hIL-10	14 ± 1.1	17 ± 1.7	1.6 ± 1.2

Purified B cells were cultured on CDw32 L cells with mAb89 (0.5 μg/ml). hIL-10 was used at 10 ng/ml as COS-7 transfectant supernatant. Control supernatant was inactive. IL-4 was used at 50 units/ml. Cells were harvested at day 12, and the percentage of immunoglobulin-secreting cells was determined by ELISPOT assays. Results are means ± SD of triplicate determinations.

entiation of activated human B lymphocytes. This demonstration of an effect of IL-10 on B cells is consistent with the reported enhancing effect of IL-10 on the viability and the Ia antigen expression of resting murine B cells (22). The proliferative effects of IL-10 on B cells activated through their antigen receptors were slightly smaller than those of either IL-2 or IL-4. However, when tested on B cells activated through their CD40 antigen, IL-10 was as potent as IL-4 when IL-2 was inactive. As cells that had been proliferating for 10 days with IL-10 and anti-CD40 expressed CD19 and CD20, we conclude that IL-10, IL-2, and IL-4 represent three well-characterized B-cell growth factors, whereas low molecular weight BCGF (23), tumor necrosis factor (24), and interferon-γ (19) may represent cofactors. The most striking activity of IL-10 was its powerful effect on the stimulation of immunoglobulin secretion by activated B cells. When B cells were activated through their antigen receptor (by SAC particles), IL-10 was much more efficient than IL-2 and IL-4. Like IL-2, it induced the secretion of the three isotypes IgM, IgA, and IgG, whereas IL-4 induced the secretion of only IgM and IgG (21). When purified B cells were stimulated in a T-cell-independent fashion through their CD40 antigen, IL-10 induced the secretion of IgM, IgG, and IgA to levels we had never seen before with any cytokine or cytokine combination (15). ELISPOT assays demonstrated that IL-10 induced virtually all anti-CD40-activated B cells to secrete immunoglobulin. However, unlike IL-4, IL-10 was not able to induce the production of IgE (15, 25). We do not know whether the IL-10-induced IgG and IgA production resulted from the expansion and differentiation of already committed precursors, from isotype switching in the naive B-cell population, or from both. Nor do we know whether IL-10 plays a role in antigen-induced and T-cell-dependent B-cell proliferation and differentiation, and whether excessive IL-10 production may be involved in diseases characterized by excessive B-cell proliferation and differentiation, such as leukemias and autoimmune diseases. It will be particularly relevant to see whether pathological situations linked to EBV infections (infectious mononucleosis, lymphomas, Duncan syndrome) result from excessive IL-10 production. In this context, it will be important to determine whether the intense B-cell proliferation induced by EBV infection could be related to the endogeneous release of IL-10, thus providing, in addition to the inhibition of interferon-γ production, another selective advantage for the survival of EBV.

The remarkable proliferation and secretion of all immunoglobulin isotypes by activated B cells in response to the combination of IL-4 and IL-10 may be consistent with the preferential role of T_H2 cells in the humoral immune response (1, 2). In keeping with this, the combination of CD40 triggering, IL-4, and IL-10 resulting in intense B-cell proliferation, immunoglobulin secretion, and isotype switching to IgE (and possibly other isotypes) may represent cardinal features of germinal centers of secondary follicles in lymphoid organs (26). As germinal centers represent the site of antibody affinity maturation through somatic mutations, one may speculate that combinations of anti-CD40, IL-4, and IL-10 may activate the somatic mutation machinery and that addition of antigen to these cultures may result in the selection of B-cell clones producing high-affinity antibodies.

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